

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicants: Miri Seiberg et al.

Attorney Docket No.: JBP-518

Serial No.: 09/698,454

Art Unit: 1617

Filed: October 27, 2001

Examiner: Yong S. Chong

For: SOY DEPIGMENTING AND SKIN CARE COMPOSITIONS

**DECLARATION OF CONNIE BAOZHEN LIN, PH.D.**

1. I, Connie Lin, am a Senior Research Fellow in the Skin Research Center, Skin biology group, at Johnson & Johnson Consumer Companies, Inc. My education includes a Ph.D. degree in Biochemistry, M.S. and B.S. degrees in Pharmaceutical Sciences. My *curriculum vitae* is attached hereto as Exhibit 1.

2. I performed a series of experiments to evaluate the different effects of soy isoflavones and non-denatured soybean extracts, as detailed in the paragraphs below. In particular, I studied the inhibitory effect of these agents on the UVB-induced activation of p38-MAP Kinase (p-p38) and on the UV-induced expression of cyclooxygenase-2 (COX-2). These studies show that non-denatured soybean extracts inhibit UVB-induced COX-2 expression and p38 activation, while soy isoflavones do not inhibit UVB-induced p38 activation and only minimally affect COX-2 expression. Therefore, I conclude that the non-denatured soybean extracts and the soy isoflavones act via different molecular pathways and that they are compound entities that are distinct from one another.

3. For the comparative studies described in paragraphs 2,5 and 6, the soy isoflavones genistin and daidzin were purchased from Sigma Aldrich (St Louis, MO), and non-denatured soybean extract was purchased from Devansoy (high sucrose powder of the whole soybean, Dupont, DE). A suspension of 2% was prepared from the non-denatured soybean extract in PBS, and was sonicated on ice by an ultra-sonic horn, for 3 times, 10 second each (Sonics,

Vibracell VC500, Sonics & Materials Inc, Newton, CT). The stock suspension was further diluted in PBS as needed.

HPLC analyses of the isoflavone content of the non-denatured soybean extract powder and the non-denatured soybean extract suspension (2%) were performed twice, independently, by Convince labs (Madison, WI). Six major soy isoflavones (daidzin, glycitin, genistin, daidzein, glycitein, and genistein) were quantified using a modified method from Klump *et al* (Klump et al., Determination of isoflavones in soy and selected foods containing soy by extraction, saponification, and liquid chromatography: collaborative study. J.AOAC Int. 2001, 84, 1865-1883). Of the six isoflavones, only daidzin, glycitin, and genistin were detected in the non-denatured soybean extract powder, with the amount of glycitin at only 5% of either the daidzin or the genistin. In the 2% soybean extract suspension, only daidzin and genistin were detectable. It was measured and calculated that 0.1% of the non-denatured soybean extract contains 0.75µg/ml of genistin and 0.6µg/ml of daidzin.

4. For the studies described below in Paragraphs 5 and 6, we used the amounts of genistin and of daidzin that are found in the 0.1% non-denatured soybean extract, and defined these amounts (0.75µg/ml of genistin and 0.6µg/ml of daidzin) as 1X. We also used genistin and daidzin at ten times higher concentrations than those found in the 0.1% non-denatured soybean extract, and defined these amounts (7.5µg/ml of genistin and 6.0µg/ml of daidzin) as 10X. The effects of these 1X and 10X genistin and daidzin concentrations were compared with the effect of 0.1% non-denatured soybean extract (which contains 1X of genistin and daidzin, respectively).

5. Human HaCaT keratinocytes (obtained from Dr. N.E. Fusenig of Heidelberg, Germany) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). HaCaT cells were plated at 20,000 cells/well in 24-well plates (Corning Incorporated, Corning, NY) and were allowed to attach overnight. Cells were then incubated in serum free DMEM for 24 hours, followed by different treatments in serum free DMEM for 24 hrs. Each treatment was performed in triplicates. Treatments include different concentrations of non-denatured soybean extract, genistin and daidzin, with or without exposure to UVB. Before UVB irradiation, the culture media were removed, followed by PBS washes. HaCaT cells were covered with a thin layer

of PBS during UVB (30 mJ/cm<sup>2</sup>) exposure. Cells that were not exposed to UVB were also covered with a thin layer of PBS without further UVB exposure. After UVB exposure, cells were further incubated in serum-free DMEM for the time indicated in each experiment.

6. HaCaT keratinocytes were treated with 0.75µg/ml (1x) and 7.5µg/ml (10x) of genistin or with 0.6µg/ml (1x) and 6µg/ml (10x) of daidzin. Cells were also treated with 0.1% of the non-denatured soybean extract. Following 24 hours of treatment, cells were extensively washed, exposed to 30 mJ/cm<sup>2</sup> of UVB, followed by incubation in culture media, and then harvested at 20 minutes post-UVB exposure for p38 activation (phosphorylation) analysis, and at 8 hours post-UVB exposure for COX-2 protein analysis. The harvested cells were lysed in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and Complete<sup>TM</sup> protease inhibitors (Roche, Indianapolis, IN)). Protein concentration was determined using the DC protein assay kit (Bio-Rad Labs, Hercules, CA). Cell lysates were adjusted to 0.5 µg/µl protein in the loading buffer (12.5% glycerol, 31.25 mM Tris-HCl, pH 6.8, 1% SDS, 0.02% Bromophenol blue, 1.25% β-mercaptoethanol). Proteins were separated on pre-cast 10% NoVex® Tris-Glycine Gels (Invitrogen, Carlsbad, CA), and were transferred to a nitrocellulose membrane (Bio-Rad labs, Hercules, CA), followed by blocking with 5% non-fat milk (Bio-Rad labs, Hercules, CA). Primary antibodies against p38, phospho-p38, and COX-2 were purchased from Cell Signaling (Danvers, MA) and used at 1:1000 dilution. β-actin antibody was purchased from Sigma, and was used at 1:5000 dilution. Secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ) and used at 1:2000 dilution. The resulting bands on the immunoblots were quantified by densitometry and normalized against β-actin (a loading control for COX-2) and against p38 (a loading control for p-p38, phosphorylated p38). The densitometric values obtained for the untreated control were defined as 100%, and the results from UVB-treated, with and without non-denatured soybean extract- or isoflavones-pretreated, in the same experiment were then compared to those of untreated controls.

7. The results of the procedure set forth in paragraphs 5 and 6 above are represented in Table 1, which summarizes the inhibitory activities of non-denatured soybean extract and of the two soy isoflavones, on UVB-induced p38 activation and on UV-induced COX-2 expression.

Treatments	No UVB exposure					UVB (30mJ/cm <sup>2</sup> )						
	Untreated control	Daidzin		Genistin		Soy	UVB only	Daidzin		Genistin		Soy
Concentrations		1x	10x	1x	10x	0.1%		1x	10x	1x	10x	0.1%
Inhibition (% relative to untreated control)												
COX-2	100	113	92	98	156	77	324	299	205	342	240	169
p-p38	100	121	261	81	264	66	548	460	435	379	366	124

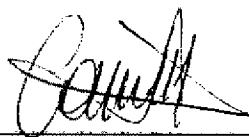
8. As shown in table 1, treatment with genistin, daidzein or the soybean extracts, without UVB exposure, somewhat enhanced COX-2 protein levels relative to untreated controls. As expected, UVB exposure led to a marked increase in COX-2 protein levels. Pretreatment with either genistin or daidzin led to a dose dependent reduction of COX-2 protein levels. However, even the 10x concentrations of either genistin or daidzin were not as potent as the 0.1% non-denatured soybean extract. UV induced COX-2 expression by 324%. This undesired induction was decreased to 169% with the non-denatured soybean extract (0.1%), while 10x of genistin or daidzin reduced these COX-2 levels only to 205 and 240% of the non UV-exposed controls). Both genistin and daidzin showed some increase in p38 phosphorylation in cells that were not exposed to UVB. UVB-induced p38 phosphorylation to 548%. Neither genistin nor daidzin, at either 1X or 10X concentration, was able to inhibit significantly the UVB-induced p38 activation. On the contrary, the non-denatured soybean extract reproducibly inhibited the UVB-induced p38 phosphorylation, from 548% (without soy) to the low level of 124%, relative to untreated controls.

9. These data suggest that the soy isoflavones only minimally contribute to the COX-2 inhibitory activity of the non-denatured soybean extract, and that, unlike the non-denatured soybean extracts, they have no effect on the p38 pathway.

10. I conclude from the results of the procedure that the whole non-denatured soybean extract inhibits two major biological pathways, the COX-2 and the p38 pathways. I also conclude from these results that genistin and daidzin, even at ten times higher concentrations than in the non-denatured soybean extracts, have minimal or no effect on these two pathways. Therefore I conclude that agents in the non-denatured soybean extract

that are different from soy isoflavones contribute to the biological activities described above of the non-denatured soybean extracts.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



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CONNIE BAOZHEN LIN

9/10/2008

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Date